AN IMPROVED METHOD FOR DETECTING NUCLEIC ACID

This application is a continuation of International application PCT/US96/18615, filed November 20, 1996, designating the U.S., which is

5 in turn a continuation-in-part of U.S. application Ser. No. 60/007,381, filed November 20, 1995, now abandoned.

BACKGROUND:

Field of the Invention

This invention is directed to methods of detecting alterations in the levels of gene expression and quantitating specific nucleotide

15 sequences in analytical samples. In particular, this invention provides a rapid analytical method based on recovery of intact nucleic acid segments that are protected from enzymatic hydrolysis through formation of hybridization dimers.

Related Art

20 The advent of molecular biology has brought with it an explosion of identified genes that exhibit developmental stage and tissue-specific expression. Identification of genes that play an important role in the etiology of human diseases, as well as genes that contribute to the basic processes underlying normal cellular function in the brain and body, have

25 become major goals of today's research in the biomedical sciences. Dynamic alterations in gene expression accompany many important changes in the health and activity of cells and tissues. Therefore, it is important to have methods that can monitor the activity of these large populations of

genes for diagnosis of diseases associated with these changes, as well as for research to identify important disease candidates and their co-varying neighbors. Any method, to be of use, should allow for high throughput and ease of performance.

5   Most methods for monitoring alterations in specific gene expression rely on RNA extraction and gel electrophoresis. Northern Blot Analysis, S1 Nuclease Protection and RNase Protection assay provide accurate data about both the size and expression of specific transcripts in question, but they are difficult to perform on multiple sample populations.

10 A major drawback of these approaches is that before performing the assay one must usually obtain a cloned fragment of DNA that is specific for the gene in question. This long sequence fragment is usually isolated by either cDNA library screening or polymerase chain reaction (PCR), and it must then be verified by DNA sequencing. In addition, the fragment of DNA

15 must contain restriction sites in the appropriate places such that the radiolabeled antisense RNAs can be made appropriate to the nucleotide sequence that is to be monitored.

       Moreover, both Northern Blot Analysis and Nuclease Protection assays rely on time consuming gel electrophoresis to distinguish

20 and display the results of the assay. In the case of the current protection assays, the quality of the results often vary from one experiment to another depending upon the target sequence, making it difficult to distinguish specific protection on a gel from a background of hybridization signals that often include protected fragments due to self-hybridization of the probe.

25   The identification and cloning approach, because of cost and time considerations, is not practical to use for detecting alterations in the

expression of multiple genes. An additional problem in these approaches is that expression can not be monitored from very small quantities of RNA. PCR-based approaches, while less sensitive to RNA limitations, are inadequate because they still rely on gel electrophoresis to confirm

5   specificity, and they have the additional problem of interpreting differences between sample sets that have been subjected to amplification. Therefore, there remains a need for improved methods of simultaneously monitoring the expression of large numbers of genes.

10   **SUMMARY OF THE INVENTION**

It is an object of this invention to provide a gene monitoring system that retains specificity while allowing for high throughput and furthermore accommodates limited tissue availability.

The method of this invention takes advantage of the high

15   specificity of nuclease protection assays using labeled oligonucleotide probes. Like those in RNase protection assays, nucleic acid probes of this invention bind to their target sequence during a hybridization reaction, and regions of unbound single stranded nucleic acid in the template and probe are subsequently digested by the addition of nuclease. An importance

20   difference, however, in the method of this invention is that specially-modified small synthetic oligonucleotides are used as probes instead of large RNA molecules produced from cloned templates using, e.g., bacterial RNA polymerases, and the hybridization signal can be detected without the use of gel electrophoresis.

25   Traditional RNase protection assays resolve protected RNA probes by PAGE electrophoresis. The quality of the results often vary from

experiment to experiment due to differences in signal-to-noise ratios. For instance, nuclease attack at multiple sites produces a heterogeneity of labeled fragments that can interfere with detection of the relevant signal and, most problematic, is the appearance of multiple bands that come from self hybridization of the probe, most often due to the formation of hairpin loops. In a particular embodiment of this invention modified oligonucleotides contain a label at the 5' end and a biotin capture group at the 3' end. Instead of PAGE analysis, protected, labeled oligonucleotide probes can be detected by streptavidin capture of the 3' biotin group on the probe. Any one cleavage event of the modified oligonucleotide will cause a total loss of detection for the molecule, thereby enhancing signal-to-noise and providing the greatest specificity of sequence detection with the protection technique.

In one embodiment, oligonucleotide sequences for the probes of this invention can be easily designed based on published sequences, and they can be produced in large quantities that are inactive and therefore RNase non-susceptible, due to the presence of a 2' t-butyldimethylsilyl protective group. To activate the ribonucleotides for biological activity, the oligonucleotide can be desilyated with tetrabutylammonium fluoride and desalted by thin layer chromatography. Small aliquots of the synthetic ribonucleotide can be stored at -80 until use. Gene expression studies using these labeled nucleotides are performed in cell lysates to minimize on RNA losses from extraction. Using this approach, gene expression can be monitored in a tissue slice of less than 500μM.

The subsequent capture and quantitation of synthetic ribonucleotide probes circumvents the necessity of using gel electrophoresis for detection while providing high throughput and specificity. Furthermore,

the method of the invention has the potential for taking a genome-based approach to mapping gene expression that can be automated to facilitate large scale analysis.

In an alternate embodiment, the label is a fluorescence label and the capture group is replaced by a second fluorescence label where the distance between the two fluorescence labels attached to the oligonucleotide probe is such that energy will be transferred from one to the other prior to fluorescence. The energy transfer will only occur when the two labels are sufficiently close to each other, usually 20 base pairs or less apart. The necessary proximity is maintained by the nucleic acid segment stretching between the labels, and the integrity of the oligonucleotide segment is therefore critical for fluorescence energy transfer. Digestion by nuclease will eliminate the energy transfer. In an assay analogous to that described above, the probes of this embodiment can hybridize to target sequences on nucleic acids in the sample, and this hybridization will protect the probe from digestion by subsequently-added nuclease. The protected probes (and the presence of corresponding target sequence in the sample) can be detected by their ability to maintain fluorescence energy transfer. Suitable fluorescent labels and suitable methods for detecting transfer of energy are well known. See, for instance, CLEGG RM, *Curr. Opin. Biotechnol.*, 1995, 6(1) p103-10; SELVIN PR, *Methods Enzymol.*, 1995, **246** p300-34; MERGNY, et al., *Biochemistry*, 1994, 33(51) p15321-8; GOHLKE, et al., *Proc. Natl. Acad. Sci. USA*, 1994, **91**(24) p11660-4; SELVIN, et al., *Proc. Natl. Acad. Sci. USA*, 1994, **91**(21) p10024-8; HIYOSHI, et al., *Anal. Biochem.*, 1994, **221**(2) p306-11; GHOSH, et al., *Nucleic Acids Res.*, 1994, 22(15) p3155-9; WU, et al., *Anal. Biochem.*, 1994, **218**(1) p1-13; and

BENSON, et al., *Nucleic Acids Res.*, 1993, 21(24) p5720-6; incorporated herein by reference.

In a related, alternative embodiment, especially suitable for probes of at least 48 bp used to detect the presence of rare mRNAs, a capture

5   group is positioned on the 5' end of the oligonucleotide and a fluorescence label (F1) is attached to the most 3' complementary base, with additional non-complementary nucleic acid sequence 3' to the end of the complementary sequence, the non-complementary sequence connecting an additional fluorescence label (F2) to the probe. The nucleotide distance

10 between F1 and F2 is adjusted such that, unless the intervening, non-complementary sequence is digested, the presence of F2 in oligonucleotide probes quenches the signal from F1. When such probes are used in assays such as those described above, oligonucleotide probes that have been protected from digestion by hybridization will be devoid of the F2 moiety

15 and will be detected by the liberation of F1 fluorescence.

In an embodiment particularly adapted to detection of very rare mRNAs (e.g., 1 - 5 pg in 10 µg of total RNA), the use of two capture groups can increase signal detection by eliminating the interference of probe which remains undigested due to enzyme inefficiency. In a typical

20 embodiment, a label is fixed to the 5' end of the oligonucleotide and a capture group (C1), preferably biotin, is attached to the most 3' complementary base. Additional nucleic acids are added to the end of the oligonucleotide, the last of which is coupled to an additional capture group (C2) that is different from C1. Reaction mixtures are first purified by

25 removing undigested oligonucleotides that contain the C2 capture group. Remaining oligonucleotides (protected by hybridization) are then purified

away from the reaction mixture by capturing the nucleic acids via the C1 capture group. Detection is then completed via the label on the 5' end of the oligonucleotide.

5   BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows percent binding to beads of an RNA oligonucleotide with and without 3' biotin label.

Figure 2 shows percent binding of probe in the presence and absence of biotin.

10   Figure 3 shows specificity of genome expression monitoring using a β-Actin antisense RNA probe with control sense RNAs.

Figure 4 shows detection of specific RNAs in lysates from brain cell cultures that contain GABA-A receptor β1 subunit transcripts.

Figure 5 shows differential expression of GABRG2 subunit 15   mRNAs in the hippocampus of 47 day old prenatally malnourished rats.

Figure 6A-6D shows, in schematic form, probe design for the detection of specific nucleic acids.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

20   Detectably-labeled oligonucleotide probes, which may be either RNA or DNA, are produced with an attaching group by which the probe may be captured, attached at some distance from the label. Thus, all probes have both a detection label and a capture group which are linked together by the oligonucleotide. For example, the probe may have a 25   detection label at the 5' end and a biotin capture group at the 3' end. The probe is incubated with the sample to be analyzed so that the oligonucleotide

hybridizes with any complementary sequence present in the sample, and then unhybridized probes are digested by nucleases specific for single-stranded nucleic acids, so that the detection label is separated from the capture group except where the probe is protected by hybridization.

5   After hybridization and digestion, the intact probes are collected. For biotin-containing probes, this can be accomplished by passing the reaction mixtures through a filter manifold containing a filter matrix with covalently-coupled streptavidin or adding streptavidin-coated beads to the reaction mixtures and then collecting the beads. Such filters or beads do not

10   bind nucleic acids if suitably pretreated, and therefore, when streptavidin captures the biotin of the probe, only full length probe, undigested because of protection by the RNA target of interest, is detected from the label on the probe's 5' end. By using this approach, it is unnecessary to perform gel electrophoresis to monitor the size of the protected fragment, and

15   furthermore, even the loss of one nucleotide of the probe could produce a species that was not detected, increasing specificity to levels greater than any technique available.

Alternative assay configurations are shown in diagrammatic form in Figure 6A-6D. In Figure 6A, a label group is attached to the 5' end

20   of the oligonucleotide and a capture group is attached to the '3 end. Sequence between these groups is complementary to the gene of interest. Enzyme digestion destroys the majority of probe, leaving behind probe that is protected by hybridization or probe that is left after due to inefficient digestion. Molecules containing either label group or capture group are not

25   recovered.

The configuration in Figure 6B shows an additional 3' capture group added to the oligonucleotide to aid in removal of all probe that is not protected by hybridization to the target sequence. Non-complementary sequence separates the two capture groups from one another. Probe

5 molecules resistant to digestion that have not hybridized to target are identified and removed via the first capture group. Probe molecules that have been protected by hybridization will not contain the second capture group. These molecules are purified via capture group 1 as in Figure 6A.

As shown in Figure 6C, for fluorescence energy transfer a

10 label group is attached to the 5' end and a different label group is attached to the 3' end. The distance between the label groups is adjusted to provide for maximum energy transfer between the two groups. Digested probes are not detected. As in Figure 6A, enzyme digestion destroys the majority of probe, leaving behind probe molecules that are protected by hybridization

15 (or background label that is left due to inefficient digestion).

In a further improvement (Figure 6D), a capture group is attached to the 5' end of the oligonucleotide and two label groups (F1 and F2) are attached to nucleotides at the 3' end of the molecule. The distance between F1 and F2 is adjusted such that F1 signal is quenched in the

20 presence of F2. Non-complementary sequence separates the F1 and F2 groups. Intact probe molecules that remain due to inefficient digestion will not be detected. Detection is specific only to the oligonucleotides that contain a 5' capture group and one label (F1).

Nucleic Acid Probes

Probes according to this invention are single stranded oligonucleotides in which at least a portion of their sequence is complementary to the sequence of some segment of a nucleic acid of

5   interest.   The   oligonucleotides   may   be   ribooligonucleotides, deoxyribooligonucleotides,   or   mixed   DNA/RNA   oligomers.   By incorporating modified deoxynucleotides (such as 5-methyl-deoxycytidine or 5-bromo-deoxyuridine) into a DNA or RNA oligomer, hybrid stability can be increased, making it possible to use shorter probes in hybridization

10   solutions   that   contain   guanidinium   or   lithium   salts.   Suitable oligonucleotides can be designed and produced for use as hybridization probes by a variety of means.   In general, the probes are synthesized chemically, preferably based upon known nucleic acid sequences.

Traditionally, those using nuclease protection assays found it

15   desirable to prepare probes that were fairly long and/or encompassed regions of the amino acid sequence which would have a high degree of redundancy in   the   corresponding   nucleic   acid   sequences.   While   it   is   generally recognized in the art that probes from about 14 to about 20 base pairs can be effective   for   hybridization,   longer   probe   sequences   were   often   found

20   necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished.   In other cases, it was found desirable to use two sets of probes simultaneously, each to a different region of the gene.   Typical probe sequences were 100-1000 nucleotides in length.   While the exact length of any probe employed is not

25   critical for the present invention, it is cheaper and easier to make shorter probes. The present method gives improved specificity with shorter probes,

and for this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 20 to about 50 nucleotides. Preferably, shorter oligonucleotide probes will be designed so that the 5' end is of relatively high G+C content. High G+C content on the

5   5' end reduces "breathing" in the hybrid that might expose the probe to hydrolysis and reduce a specific signal. For hybridization in guanidium or lithium salts, probes will be preferably at least 40 base pairs.

Probes having the desired oligonucleotide sequence can be constructed synthetically, based on known DNA or amino acid sequences,

10   or isolated by one of several approaches using well known methods. The basic strategies for preparing oligonucleotide probes and DNA libraries are well known to those of ordinary skill in the art. See, e.g., Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989); B. Perbal, "A Practical Guide To Molecular Cloning" (1984), Edge (1981) *Nature* **292**:756;

15   Nambair, et al. (1984) *Science* **223**:1299; and Jay, et al. (1984) *J. Biol. Chem.*, **259**:6311. Suitable RNA oligomers, for RNase protection assay, or DNA oligomers, for S1 analysis, may be prepared on the ABI 392 DNA/RNA synthesizer.

Probes according to one embodiment of this invention are

20   connected to both a detectable label and one or more capture groups. By the connections of the label and capture group to the oligonucleotide, the label and capture group remain coupled so long as the oligonucleotide is intact, and immobilization of the capture group serves to anchor the label to which it is indirectly coupled. However, upon digestion of the oligonucleotide, the

25   label is no longer coupled to the capture group, and the label may then be readily separated from the capture group once the latter is captured, for

example by binding it to a suitable moiety immobilized on a solid substrate.

Any group susceptible to capture may be attached to the oligonucleotide. Suitable capture groups are usually one member of a specific binding pair, such as biotin and avidin or streptavidin, an antigen and its antibody, a protein containing at least the F_C portion of an immunoglobulin molecule and staphlococcal protein A, a lectin and its corresponding carbohydrate moiety, or similar pairs. Methods for connecting capture groups to nucleotide residues of the oligonucleotide will depend on the specific capture group and the nature of the nucleotide residue to which it is being attached. Such methods are well known and selection of a suitable method can be readily made by the skilled artisan. See, e.g., Sambrook, et al. (1989), Perbal, et al. (1984), and Ausubel, et al., eds., "Current Protocols in Molecular Biology" (1989).

The label is detectable by physical or chemical means, such as colorimetric, chemiluminescent or fluorescent methods or radio detection. Typical labels include radiolabels incorporated in one or more of the nucleotide residues of the oligonucleotide or in a moiety attached to one of the residues, a fluorescent group attached to one of the residues, or a member of a specific binding pair different from the capture group, where the second member of the specific binding pair is attached to a detectable moiety such as an enzyme catalyzing a chromogenic or fluorogenic reaction either directly or indirectly. Any method of connecting the label to the oligonucleotide portion of the probe may be used, so long as the connection survives under the conditions used in the hybridization step as well as the conditions of the digestion if enzyme is omitted. A variety of suitable labels

are in routine use for analysis of various nucleic acid materials, and selection of a suitable label and incorporation of the label in the probe is within the skill of the art.

The detectable label and the capture group(s) may be connected to the oligonucleotide at any point on the oligonucleotide and by any suitable method of connection, so long as coupling between the label and capture group (or between different capture groups) is destroyed by digestion of the oligonucleotide. Clearly the label and the capture group cannot be attached to the same nucleotide residue, and usually there will be several residues of the oligonucleotide between the label and the capture group. Increasing the distance between connection points to the oligonucleotide for the capture group and the label will tend to increase sensitivity of the assay. (This does not preclude the presence of an additional label attached to the same nucleotide residue as the capture group for control purposes.) Where fluorescence energy transfer between two fluorescent labels is critical to the detection method, the distance between the two labels will, of course, be designed to favor energy transfer or quenching, in accordance with the method.

Preferably, oligonucleotides are made to contain a biotin group on the 3' end. This may be accomplished through the use of a CPG Biotin-ON column or CPG Amino-ON column (Clonetech), although the skilled artisan will be aware of numerous other methods of attaching capture groups to a nucleic acid sequence. After conjugating to the capture group, RNA oligomers are desilated overnight and gel purified. Conjugated DNA oligomers are similarly deprotected overnight and also gel purified.

Oligonucleotides conjugated to capture groups at the 3' end can be subsequently labeled at the 5' end with either γ^{32}PdATP or γ^{35}SdATP in a reaction that is catalyzed by T4 polynucleotide kinase. For example, probes to monitor rare mRNA of specific interest may be labeled with γ^{32}P

5 while probes to monitor abundant internal standards, such as β-actin, are labeled with γ^{35}S. Alternatively, oligonucleotide probes containing a fluorescent tag at the 5' end and a biotin group at the 3' end could also be used in this detection assay. Multiple fluorescent tags on different probes allows the expression of many genes to be monitored together in one sample,

10 in addition to internal standards. Alternative labels that facilitate enhanced detection are also suitable. See, e.g., U.S. Pat. Nos. 5,312,922 and 5,089,423, incorporated herein by reference. In the fluorescence energy transfer assay, the different fluorescence labels are chosen such that the proximity of labels produces either energy transfer or energy depletion.

15 Fluorescent labels suitable for proximity dependent energy transfer or fluorescent quenching can be selected from probes known to provide these characteristics (see Clegg, 1995; Selvin, 1995: Mergny et al., 1994; Gohlke, 1994; Selvin, 1994; Hiyoshi et al., 1994; Ghosh et al., 1994; Wu et al., 1994; and Benson et al., 1993).

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Sample Preparation

The method of this invention may be used to detect and/or quantitate the amount of a particular nucleotide sequence in single stranded regions of nucleic acid in any sample. As discussed herein, nucleic acid

25 containing the particular sequence complementary to the sequence of the probe is the target nucleic acid, and the sequence complementary to the

oligonucleotide of the probe is the target sequence. While the method of this invention may be used to detect the presence of specific nucleotide sequences in any single stranded nucleic acids of a sample, the target nucleic acid is usually messenger ribonucleic acid (mRNA). In particular, the

5   method of this invention is useful for monitoring the appearance and disappearance of mRNA encoding various proteins as a result of embryonic development or cellular signaling in response to hormonal changes.

Assay of RNA according to earlier methods (see, e.g., Ausubel, et al., eds., "Current Protocols in Molecular Biology," Volume 1,

10   Chapter 4, 1989) required isolation of the RNA from cellular components, particularly RNases, and specific sequences in isolated single stranded RNA can be detected and quantitated with probes according to this invention. While purified RNA samples may be assayed by the present method, the method of the present invention can also be used to detect mRNA in cell

15   lysates from cell culture or tissue samples without the need for extensive prior purification of the nucleic acids. The present method thus permits rapid screening of large numbers of cell samples, which would be unfeasible using earlier methods.

For example, mRNA expression by cells in cell culture may

20   be determined by obtaining an aliquot of cells from the culture and making the nucleic acids available for hybridization and enzymatic digestion by simply lysing the cells. In one embodiment, a cell culture sample is collected by centrifugation, the supernatant removed from the centrifuge tube, and the tube then vortexed to distribute the cells as a layer on the inside

25   wall. This layer is then dissolved, for example, at a concentration of 10^5 cells/ml in lysis buffer solution (4 M guanidine thiocyanate, 25 mM sodium

citrate and 0.5% sarcosyl) and vortexed. The resulting lysate may be used directly in assays according to this invention.

For tissue samples, lysis of the tissue section to release RNA may be all that is necessary. For example, the tissue may be placed in lysis buffer, such as the solution described above, at a concentration of 100-250 mg/ml and then homogenized. After lysis of the cells or tissue sections, lysates can be stored at -20°C or used immediately in the hybridization reaction.

Hybridization Assay Procedures

The assay method of this invention is carried out by contacting the probe, including a single stranded oligonucleotide connecting detectable label and one or more capture groups, with the target nucleic acid under conditions that promote hybridization, then digesting any probe oligonucleotide that remains in the single stranded state. Preferably, the assay protocol includes both positive and negative controls. Most preferably the positive controls are internal, wherein multiple probes are added to the sample, at least one of which is complementary to a sequence that is known to be present in the sample or is exogenously added to the sample before the hybridization step. Usually the negative controls are external, i.e., separate samples which may contain the positive control sequence, but are known not to contain the target sequence.

The conditions of temperature, chemical environment, etc., in the sample must be adjusted so that the target nucleic acid material can undergo hybridization with the probe oligonucleotide and also to permit enzymatic degradation by enzymes specific for single stranded nucleic acids. These conditions are well known to the skilled artisan and suitable

conditions are exemplified below (see also Ausubel, et al., eds., "Current Protocols in Molecular Biology," Volume 1, Chapter 4, Sections 4.6 and 4.7, 1989, incorporated herein by reference). Incubation to permit hybridization is usually from about one hour to about 16 hours (overnight), although

5   shorter or longer times may be used so long as sufficient hybridization occurs to protect the oligonucleotide portion of a detectable amount of the probes from subsequent digestion.

Hybridization and digestion may occur simultaneously, for instance when endogenous RNases are used, so long as a detectable quantity

10   of probe molecules are protected from digestion, and the quantity is proportional to the amount of the target sequence present in the sample. However, the hybridization and digestion are usually carried out sequentially. In either case, the sample must be incubated for sufficient time to allow the remaining single stranded oligonucleotide portion of the probe

15   molecules to be hydrolyzed by nucleases which are selective for single stranded nucleotides relative to double stranded nucleotides. Enzymes of greater selectivity, as reflected in higher relative rates of hydrolysis for single stranded nucleic acids over double stranded nucleic acids, is clearly preferable to preserve a detectable amount of hybridized probe while

20   digesting essentially all single stranded probe (to leave no more than background levels of label coupled to capture group by single stranded oligonucleotide). While conditions and probe concentration may permit endogenous RNase in the sample to digest the residual single stranded probe molecules, it is usually preferable to add an exogenous nuclease. Typical

25   nucleases are S1 nuclease when the probe oligonucleotide is DNA and RNase T1 and/or RNase A when the probe oligonucleotide is RNA.

Following the digestion step, the capture group (along with any intact probes still attached thereto) is separated from the reaction mix. Usually, the separation is accomplished by binding the capture group to a solid material which may be porous or non-porous particles, including

5   magnetic beads, a filter, such as a membrane filter, or a solid surface, such as plastic plates, glass plates, or porous plates. Typically the capture group is one member of a specific binding pair, such as biotin-streptavidin, antigen-antibody, antibody-protein A, etc., and the other member of the binding pair is conjugated to a suitable surface. The post-digestion reaction

10   mix is contacted with the surface conjugated with members of the specific binding pair which bind the capture group, immobilizing it on the surface when the rest of the reaction mix is removed (e.g., by washing the surface). The amount of intact probes which survived digestion can be determined by measuring the amount of detectable label attached to the surface by coupling

15   through intact oligonucleotides connected to the immobilized capture group, using methods for quantitating detectable label attached to a surface which are well known in the art. In the event that background levels of undigested probe are too great to detect rare mRNAs, two capture groups may be coupled to nucleotides in the oligomer. The most 3' capture group will allow

20   a pre-separation of background probe from relevant hybridization signal. The final capture and detection will be as previously described.

Detection

Radioactive labels may be detected and/or quantitated using a scintillation counter, Phosphoimager (densitometer) or autoradiography.

25   Alternatively, a fluorescence detector can be used if a fluorescent tag is attached to the oligonucleotide during synthesis. After separation, detection

of flourescent labels may be monitored in 96 well plates using a fluorescent plate reader. Multiple fluorescent tags on different probes allows the expression of many genes to be monitored together in one sample. For other labels, the appropriate detection system will be readily apparent to the

5 skilled worker.

Exemplary Assay Procedure

In a typical assay, the radiolabeled probe, with oligomeric RNA for RNase protection or oligomeric DNA for S1 protection, is diluted in lysis buffer solution at 10^4-10^5 cpm/5μl. 5μl of diluted probe is then

10 aliquoted into each of a set of 1.5ml tubes. 45μl of cell lysate is added to each tube and mixed by gentle vortexing. Control reactions contain lysate from a tissue source known to not express the message of interest. The reactions are then incubated overnight at 37°C (this temperature varies with the GC content of the probe). Selection of the nuclease will, of course,

15 depend on whether the probe is RNA or DNA.

RNase Protection: 500 μl of RNase solution (450 μl dH$_2$O, 50 μl of 10X RNase buffer (500 mM Tris, pH7.5, 4M NaCl, 100 mM EDTA) and 1 μl of T1 (RNase T1, 100 U) are added directly to the hybridization reactions. Separate control reactions for positive controls do not contain

20 nuclease.

S1 Protection: 40 μl of 2XS1 digestion buffer (0.56M NaCl, 0.1M NaOAc, 9 mM ZnSO$_4$ and 250 U of S1 nuclease are added directly to the hybridization reactions. Positive control reactions do not contain nuclease.

25 Reactions are then gently vortexed and incubated at 37°C for 60 min. After the reaction mixture has equilibrated to room temperature, one

of two methods may be used to capture intact probe containing a 5' radiolabel and a 3' biotin group: 25 μl of equilibrated streptavidin magnetic beads are added to the reaction mixture. After 10 min of incubation at room temperature, protected probe can be recovered by magnetic separation from

5   the reaction mixture followed by immobilization on filter paper, either through a slot blot or filter disk apparatus, and analyzed subsequently by phosphoimaging or scintillation counting. In addition, biotinylated probe can be captured directly onto streptavidin containing filters without the use of magnetic beads.

10   Detection: Radioactivity on the filter can then be monitored using a Phosphoimager for $\gamma^{32}P$ and ^{35}S detection. Alternatively, a fluorescence detector can be used if a fluorescent tag is incorporated at the 5' end of the RNA oligomer during synthesis. After separation, detection can then be monitored in 96 well plates using a fluorescent plate reader.

15   Multiple fluorescent tags on different probes allows the expression of many genes to be monitored together in one sample.

Customarily, the expression of a gene transcript common to all cells (i.e., a housekeeping gene) is monitored in addition to the experimentals in order to control for differences across samples and for

20   pipette loading errors. As with all assays that monitor changes in gene expression, it is important to first demonstrate that the expression of the housekeeping gene does not change with the condition under study.

Probes having 5' label and 3' biotin or amino group (or other similar label and capture group) can be used in a variety of different methods

25   (S1 nuclease protection, in solution hybridization, standard RNase protection with large RNA probes) modified to rely on recovering the probe from a

reaction mixture (for example by streptavidin filtration) in order to quantitate the specificity of hybridization without gel electrophoresis.

Structural Characterization of Macromolecules

This invention also provides a method for characterizing the tertiary structure of a protein or other macromolecule, especially a method for measuring distances between epitopes on the surface of the macromolecule. An antibody specific for one epitope on a macromolecule is conjugated with an oligonucleotide having a capture group distal to the antibody. A second antibody, specific for another epitope distinct from the first epitope, is conjugated to an oligonucleotide complementary to the oligonucleotide conjugated to the first antibody and having a detectable label distal to the second antibody.

The conjugated antibodies are used to characterize the macromolecular structure by binding the antibodies to epitopes on the macromolecule and allowing hybridization between the complementary oligonucleotides if the antibodies (and therefore the epitopes they bind to) are positioned in close enough proximity to permit it. The antibody-macromolecule complex is then subjected to nuclease digestion that will digest the oligonucleotides (and release the capture group and label) unless hybridization has occurred. Capture of the antibody-macromolecule complex, and detection of the detectable label in association with the complex (but not with uncomplexed antibodies), confirms the proximity of the two epitopes on the macromolecule under conditions of the assay.

Suitable conditions can be readily selected by the skilled artisan, guided by the conditions for hybridization, digestion, and detection provided herein, as can suitable oligonucleotides, capture groups, and

detectable labels. It will be readily apparent to the skilled artisan that if the detectable label is a fluorescent label, a second fluorescent label can be substituted for the capture group and detection of hybridization accomplished by detecting fluorescent transfer. Adoption of the method

5   using a second capture group to enhance sensitivity is also easily within the skill of the artisan.

Immunoassays

This invention also provides improved immunoassay methods

10   for detecting a particular protein or other antigen in a sample comprising mixing a sample containing the antigen with a primary antibody that is conjugated to a first nucleic acid sequence under conditions where antigen-antibody complexes form, then mixing the sample with an oligonucleotide probe according to this invention complementary to the said first nucleotide

15   sequence conjugated to the primary antibody, and incubating for a time sufficient to permit hybridization of complementary nucleic acid sequences to for nucleic acid duplexes; and subsequent to the hybridization, adding to the sample a nuclease which specifically digests the probe unless the probe is part of a nucleic acid duplex. Detection of the probe bound to the

20   complementary oligonucleotide (which is conjugated to the antibody) as described herein, provides a method of detecting the antibody, and therefore the antibody-antigen complex in which it is bound.

Such assays can alternatively employ the process described above, based on hybridization of two oligonucleotides brought into

25   proximity by proximal binding of two antibodies to which the oligonucleotides are attached, and digestion of unhybridized

oligonucleotides. It will be readily apparent that a particular embodiment of the general process occurs when one oligonucleotide is conjugated to an antibody that specifically binds the other antibody (thereby ensuring proximity of the two oligonucleotides conjugated to the two antibodies).

5   Use of this process to detect binding of a secondary antibody to a primary antibody specific for a particular analyte by a procedure including hybridization, digestion and detection as described herein is contemplated by the present invention. Such an analytical procedure is particularly attractive to confirm binding of the secondary antibody to the primary

10   antibody, which may be used, for example, to provide an internal experimental control. Similar assay procedures may, of course, employ specific binding pairs with characteristics analogous to antibody binding.

EXAMPLES

15   In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

Example 1.   RNA capture and detection

20   *Preparation of Probes*

RNA having a sequence complementary to the sequence to be detected   is   prepared   on   the   ABI   392   DNA/RNA   synthesizer. Oligoribonucleotides ($\geq$48bp) are made to contain a biotin group or amino group on the 3' end. This is accomplished through the use of a CPG Biotin

25   column (Clonetech). RNA oligomers are desilylated overnight and gel purified. Oligoribonucleotides are subsequently labeled at the 5' end with

either $\gamma^{32}PdATP$ or $\gamma^{35}SdATP$ in a reaction that is catalyzed by T4 polynucleotide kinase. Probes having sequence complementary to rare mRNA of specific interest are labeled with $\gamma^{32}P$ while probes complementary to abundant internal standards are labeled with $\gamma^{35}S$. The

5 internal standards may be RNA common to most cells, such as β-actin, or exogenously added RNA or single stranded DNA having sequences that are not expected in the sample. Usually the sequences complementary to control probes will be present in substantial excess.

Cell lysates

10   The cell culture sample is collected by centrifugation and the supernatant is removed. The tube is then vortexed to distribute the cells as a layer on the inside wall. This layer is then dissolved at a concentration of 10^5 cells/ml in lysis buffer solution (4 M guanidine thiocyanate, 25 mM sodium citrate and 0.5% sarcosyl) and vortexed. At this point lysates can be

15 stored at -20°C or used immediately in the hybridization reaction.

Hybridization Assay

      The radio labeled RNA probe is diluted in lysis buffer solution at 10^4-10^5 cpm/5μl. 5μl of diluted probe is then aliquoted into each 1.5ml tube. 45μl of cell lysate is added to each tube and mixed by gentle

20 vortexing. Negative control reactions contain lysate from a cell or tissue source known to not express the message of interest. The reactions are then incubated overnight at 37°C (this temperature varies with the GC content of the probe).

      500 μl of RNase solution (450 μl dH2O, 50 μl of 10X RNase

25 buffer (500 mM Tris, pH7.5, 4M NaCl, 100 mM EDTA) and 1 μl of T1 (RNase T1, 100 U) are added directly to the hybridization reactions. Control

reactions do not contain nuclease. Reactions are then gently vortexed and incubated at 37°C for 60 min. After the reaction mixture has equilibrated to room temperature, one of two methods is used to capture intact probe containing a 5' radiolabel and a 3' biotin group: 25 µl of equilibrated

5 streptavidin magnetic beads are added to the reaction mixture. After 10 min of incubation at room temperature, protected probe is recovered by magnetic separation. Activity is monitored by scintillation counting. Probes captured on the beads can also be separated from the reaction mixture by immobilization on filter paper, either through a slot blot or filter disk

10 apparatus, and analyzed subsequently by phosphoimaging or scintillation counting. In addition, the protected biotinylated probe can be captured directly onto streptavidin-containing filters without the use of magnetic beads.

Figure 1 shows percent binding to beads of an RNA

15 oligonucleotide with and without 3' biotin label. Avidin coupled magnetic beads were incubated in the presence of radiolabeled oligonucleotide that did and did not possess a biotin group at the 3' end. Percent binding was determined by measuring the amount of radioactivity associated with the beads.

20 Figure 2 shows percent binding of probe in the absence and presence of biotin. A molar excess of biotin (5.2 nmol) over magnetic beads coupled to streptavidin successfully competed for the binding of the biotinylated probe. The results of this experiment demonstrate that binding of the probe to streptavidin beads is dependent on the presence of the 3'

25 biotin capture group in the oligonucleotide.

Figure 3 shows specificity of genome expression monitoring using a β-Actin antisense RNA probe with control sense RNAs. Percent protection was monitored using a tenfold excess of probe over target sense RNA. Significant protection was monitored only when the target contained

5   sequence complementary to the probe.

Figure 4 shows detection of specific RNAs in lysates from brain cell cultures that contain GABA-A receptor β1 subunit transcripts. Lysates were prepared from one 100mm dish containing cultured cells from either rat hindlimb or rat neocortex. Significant detection of the neutral

10   specific β1 subunit transcript was detected in rat neocortex.

Figure 5 shows differential expression of GABRG2 subunit mRNAs in the hippocampus of 47 day old prenatally malnourished rats. Where A41 represents well nourished rats and B41 malnourished, detection of γ2 transcripts in RNAs extracted from hippocampal tissue indicates a

15   significant increase of expression in the brain of malnourished animals.

Example 2. Detection with Fluorescent Labels

The procedure is identical to that of Example 1, except oligonucleotide probes containing a fluorescent tag at the 5' end and a biotin

20   group at the 3' end are used in this detection assay. Detection of fluorescent probes may be monitored in, for example, 96 well plates using a fluorescent plate reader or CCD camera.

Synthetic RNA can also be produced using standard methods for the production of Riboprobes. These Oligonucleotides are then tagged

25   with biotin and labeled at the 5' end with detectable labels as described above.

In assays that employ fluorescence energy transfer, reaction mixtures are subjected to enzymatic digestion. For small probes, the amount of energy present at a particular region of the spectrum is monitored using standard procedures. For the detection of rare mRNAs, the protected probe

5 is recovered after digestion using a capture procedure as previously described and the presence of fluorescence from the F1 label is detected using standard methods. Control assays are performed to measure the integrity of the RNA probe. The fluorescence from a fully intact probe is quenched and therefore will not be detected until the F2 label is removed by

10 digestion.

Example 3.   RNA detection using DNA probes

Probes

Short DNA oligonucleotides are prepared on the ABI 392

15 DNA/RNA synthesizer. Oligodeoxyribonucleotides ($\geq$48bp) are made to contain a biotin group on the 3' end. This is accomplished through the use of a CPG Biotin column (Clonetech). DNA oligomers are deprotected overnight and gel purified. Oligodeoxyribonucleotides are subsequently labeled at the 5' end with either γ^{32}PdATP or γ^{35}SdATP in a reaction that is

20 catalyzed by T4 polynucleotide kinase. Probes which hybridize with rare mRNA of specific interest are labeled with γ^{32}P while probes which hybridize with internal standards are labeled with γ^{35}S. Usually the internal standards are segments of sequences found in abundance, such as β-actin. Oligonucleotide probes containing a fluorescent tag at the 5' end and a biotin

25 group at the 3' end can also be used in this detection assay.

Hybridization Assay

A tenfold molar excess of radiolabeled DNA probe and 2-20 µg of RNA extracted from the tissue of interest are added to 10 µl of hybridization buffer (80% deionized Formamide/100mM Sodium Citrate pH.

5   6.4/300 mM Sodium Acetate pH. 6.4/1mM EDTA). Negative control reactions contain RNA from a tissue source known to not express the message of interest. The reactions are then incubated overnight at 37°C (this temperature varies with the GC content of the probe).

10 µl of 2XS1 digestion buffer (0.56M NaCl, 0.1M NaOAc,

10   9 mMZnSO$_4$ and 250 U of S1 nuclease are added directly to the hybridization reactions. Positive control reactions do not contain nuclease. Reactions are then gently vortexed and incubated at 37°C for 60 min. After the reaction mixture has equilibrated to room temperature, one of two methods is used to capture intact probe containing a 5' radiolabel and a 3'

15   biotin group: 25 µl of equilibrated streptavidin magnetic beads are added to the reaction mixture. After 10 min of incubation at room temperature, protected probe is recovered by magnetic separation. Activity is monitored by scintillation counting. Captured beads can also be separated from the reaction mixture by immobilization on filter paper, either through a slot blot

20   or filter disk apparatus, and analyzed subsequently by phosphoimaging or scintillation counting. In addition, biotinylated probe can be captured directly onto streptavidin containing filters without the use of magnetic beads.

Example 4. Antigen Detection using Conjugated Antibodies.

A primary antibody is conjugated to a nucleic acid that contains sequence complementary to the especially prepared probe (prepared by the method of Sano, Smith, and Cantor, *Science* **258**:5079, 1992). The antibody is mixed with either an antigen solution containing lysed cells or a solution containing solubilized cells at 20 - 200 ng/ml antigen to 1 -10 μg/ml antibody. Solution is incubated for 30 min. Antigen-antibody complexes are separated from unbound antibody. Probe complementary to a region of the oligonucleotide coupled to the antibody is then added to the reaction mixture containing antigen-antibody complexes and hybridization and digestion takes place as described in the above examples. Hybridization is then detected using a method that is dependent on the probe configuration, i.e. one or more capture groups and fluorescence energy transfer (see Figure 5).

Antigen detection can also be carried out using DNA-coupled antibodies that are affixed to a surface, such as a magnetic bead or a microtiter well plate. In addition, the oligonucleotide attached to the antibody may contain one or more fluorescence labels that can interact with one or more fluorescence labels on the detection probe. Hybridization after digestion would then be detected by a fluorescence energy transfer or liberation of quenching due to the proximity between the two protected oligonucleotides.

Example 5. Allelic discrimination.

A probe, in a configuration as described in Diagram 1, is generated that contains one or more mutations in the nucleic acid sequence specific to a previously identified variant or suspected variant.

Hybridization to DNA or RNA is performed as previously described. Detection of the probe indicates the presence of the variant form of nucleic acid sequence. Control reactions include the addition of wildtype DNA oligonucleotide (containing a majority of complementary sequences to the

5   probe) in the presence and absence of the target of interest in order to verify that the probe is only detecting the variant. Variant alleles can alternatively be detected by the loss of probe signal when the probe contains only sequence of the wildtype.

Example 6

10   Procedure is identical to Example 1 except that an oligoribonucleotide is synthesized to contain two capture groups that are separated by non-complimentary sequence. The first capture group is biotin and the second is an amino group at 3' end of the oligoribonucleotide. After hybridization any probe remaining due to inefficient digestion is removed by

15   covalent coupling to tosylactivated magnetic beads (Dynabeads) Hepburn et al., *American Biotechnology Laboratory*, September 1992: 44-45. The unbound mixture containing the hybridization is recovered using streptavidan purification as described above.

Example 7

20   Procedure is identical to Example 6 except that the probes are DNA rather than RNA molecules and hybridization occurs as described in Example 3.

Example 8. Detection with Fluorescent Labels

        The procedure is identical to that of Example 3, except

25   oligonucleotide probes containing a fluorescent tag at the 5' end and a biotin group at the 3' end are used in this detection assay. Detection of fluorescent

probes may be monitored in, for example, 96 well plates using a fluorescent plate reader or CCD camera.

Synthetic DNA can also be produced using standard methods for the production of DNA probes. These oligonucleotides are then tagged 5 with biotin and labeled at the 5' end with detectable labels as described above.

In assays that employ fluorescence energy transfer, reaction mixtures are subjected to enzymatic digestion. For small probes, the amount of energy present at a particular region of the spectrum is monitored using 10 standard procedures. For the detection of rare mRNAs, the protected probe is recovered after digestion using a capture procedure as previously described and the presence of fluorescence from the F1 label is detected using standard methods. Control assays are performed to measure the integrity of the probe. The fluorescence from a fully intact probe is 15 quenched and therefore will not be detected until the F2 label is removed by digestion.

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, 20 advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in medicine, clinical chemistry, immunology, molecular 25 biology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. The disclosures in all publications and patent applications cited herein are incorporated by reference to the same extent as

5   if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.